



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 9/127		A1	(11) International Publication Number: WO 95/12387 (43) International Publication Date: 11 May 1995 (11.05.95)
(21) International Application Number: PCT/US94/12350 (22) International Filing Date: 3 November 1994 (03.11.94)		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 08/148,099 5 November 1993 (05.11.93) US			
(71) Applicant: AMGEN INC. [US/US]; Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US).			
(72) Inventor: COLLINS, David, Sammuel; 127 McAfee Court, Thousand Oaks, CA 91360 (US).			
(74) Agents: ODRE, Steven, M. et al.; Amgen Inc., Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US).			

(54) Title: LIPOSOME PREPARATION AND MATERIAL ENCAPSULATION METHOD

(57) Abstract

The present invention relates to a method of producing liposomes useful for encapsulating and delivering a wide variety of biologically active materials. The invention provides liposomes and a production method which is simple, feasible and inexpensive for the large-scale commercial manufacturing of liposomes and encapsulated materials. The method involves the formation of a liposome dispersion in the absence of an organic solvent or detergent, one or several cycles of freezing and thawing the liposomes, and dehydration of the liposome dispersion to form a lipid powder. When desired, the lipid powder is hydrated in the presence of the biologically active material whereby the material is encapsulated in reconstituted liposomes. The method can also include combining the liposome dispersion with a bulking agent prior to the dehydration and formation of the lipid powder. The addition of the bulking agent facilitates the handling of the lipid powder as well as its rapid dispersal upon hydration.

- 1 -

**LIPOSOME PREPARATION AND MATERIAL ENCAPSULATION
METHOD**

5

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to liposomes, and more particularly to a method of 10 producing liposomes useful for encapsulating biologically active materials. The liposomes are, therefore, useful in applications such as *in vivo* drug delivery and gene therapy and as diagnostic agents.

15

Description of the Background

Liposomes are microscopic vesicles, generally spherically shaped, formed from one or more lipid walls. The walls are prepared from lipid molecules, 20 which have the tendency both to form bilayers and to minimize their surface area. The lipid molecules that make up a liposome have hydrophilic and lipophilic portions. Upon exposure to water, the lipid molecules form a bilayer membrane wherein the 25 lipid ends of the molecules in each layer are directed to the center of the membrane, and the opposing polar ends form the respective inner and outer surfaces of the bilayer membrane. Thus, each side of the membrane presents a hydrophilic surface 30 while the interior of the membrane comprises a lipophilic medium.

Liposomes can be classified into several categories based on their overall size and the nature of the lamellar structure. The classifications 35 include small unilamellar vesicles (SUV), multilamellar vesicles (MLV), large unilamellar

- 2 -

vesicles (LUV), and oligolamellar vesicles. SUVs range in diameter from approximately twenty to fifty nanometers and consist of a single lipid bilayer surrounding an aqueous compartment. A characteristic 5 of SUVs is that a large amount of the total lipid, about 70%, is located in the outer layer of the bilayer. The most frequently encountered and easily prepared liposomes are the multilamellar vesicles. Where SUVs are single compartment vesicles of a 10 fairly uniform size, MLVs vary greatly in diameter up to about 30,000 nanometers and are multicompartamental in their structure wherein the liposome bilayers are typically organized as closed concentric lamellae with an aqueous layer separating each lamella from 15 the next. Large unilamellar vesicles are so named because of their large diameter which ranges from about 600 nanometers to 30 microns. Oligolamellar vesicles are intermediate liposomes having a larger aqueous space than MLVs and a smaller aqueous space 20 than LUVs. Oligolamellar vesicles have more than one internal compartment and possibly several concentric lamellae, but they have fewer lamellae than MLVs.

A variety of methods for preparing liposomes are known in the art, several of which are described in 25 *Liposome Technology* (Gregoriadis, G., editor, three volumes, CRC Press, Boca Raton 1984) or have been described by Lichtenberg and Barenholz in *Methods of Biochemical Analysis*, Volume 33, 337-462 (1988). Liposomes are also well recognized as useful for 30 encapsulating biologically active materials. Preparation methods particularly involving the encapsulation of DNA by liposomes, and methods that have a direct application to liposome-mediated transfection, have been described by Hug and Sleight 35 in *Biochimica and Biophysica Acta*, 1097, 1-17 (1991).

- 3 -

When liposomes form, solvent and solute molecules become trapped in their lumen. The volume encapsulated, or capture volume, is dependent on the size of the liposomes, the lipid composition of the vesicles and the ionic composition of the medium.

5 The fraction of the solvent entrapped by liposomes is defined as the encapsulation or entrapment efficiency and is proportional to both lipid concentration and vesicle radius. The fraction of solute sequestered

10 inside the liposomes is generally directly proportional to the fraction of solvent entrapped.

Although the encapsulation of biologically active materials in liposomes has significant potential for delivering such materials to targeted

15 sights in the human body, the production of encapsulated materials on a commercially feasible scale has been a problem. In order for liposomes to be used more widely for therapeutic purposes, it is desirable that the preparation process satisfy the

20 following standards:

- 1) a high degree of encapsulation can be attained;
- 2) organic solvent or detergent can be completely removed from the final product or their use avoided;
- 3) the final product is obtainable by a simple

25 procedure;

- 4) preparation can be carried out on a large scale;
- 5) the stability of the liposomes supports an appropriate storage period; and
- 6) the encapsulated material is not partially or

30 completely denatured or inactivated during liposome production or encapsulation.

A method for preparing liposomes with a water-soluble, biologically active compound using lyophilization is disclosed in U.S. Patent No. 35 4,311,712 to Evans, et. al. Evans, et. al., however, state that their methods are not

- 4 -

particularly suitable for aqueous soluble materials. Moreover, the disclosed method of preparation requires the mixture of the biologically active material with an organic solvent. Felgner, et al. 5 (EP 172 007 B) also describe a technique incorporating an organic solvent. One object of the present invention is to avoid the use of organic solvents or detergents in forming the liposomes because these substances are difficult to remove, 10 present health hazards or interact unfavorably with the biologically active molecules to be encapsulated.

It is also known that liposomes and their contents may be relatively unstable in aqueous dispersion. Accordingly, an attempt to increase the 15 relatively short storage life of certain liposomal products by dehydrating the dispersion has been the focus of several liposome preparation methods. For example, the aqueous dispersion of encapsulated material is lyophilized to form a stable powder which 20 can be stored for a long period and from which, with an aqueous medium, a liposome dispersion can be reconstituted (see Schneider, et. al. in U.S. Patent No. 4,229,360). U.S. Patent No. 4,515,736 (D. Deamer) also describes an encapsulation method in 25 which liposome dispersions are dried in the presence of the material to be encapsulated. As the solution is dried to a highly viscous concentrated mixture, the individual liposomes fuse to form multilamellar structures which capture the material to be 30 encapsulated between the lipid lamellae. Upon rehydration, lipid vesicles form which encapsulate the material. Crowe, et al. (U.S. Patent 4,857,319) describe a method for preserving liposomes involving freeze drying a mixture containing the lipid 35 vesicles, the material to be encapsulated and a disaccharide preserving agent. Each of these methods

- 5 -

requires that the biologically active material be subjected to the lyophilization procedure. In contrast, it is a further object of the present invention to encapsulate a material while avoiding 5 the need to subject that material to such rigorous manipulations as lyophilization, thereby decreasing the possibility of physically inactivating or degrading the material to be encapsulated.

Mayer, et. al. (U.S. Patent No. 5,077,056) 10 describe a liposome preparation method involving the use of small ions to produce gradients which enhance the retention of charged biologically active agents, and optionally involving subjecting the liposome and biologically active agents to a freeze-thaw process 15 during the encapsulation procedure. The method (as further described by Mayer, et al in *Biochimica Biophysica Acta* 817: 193-196; 1985) produces "freeze and thaw multilamellar vesicles" (FATMLV). The FATMLV method requires that freezing and thawing be 20 done in the presence of the material to be entrapped. In addition, while high capture of small ions ($^{22}\text{Na}^+$ and Mg^{2+}) was demonstrated using the FATMLV method, no encapsulation of macromolecules was achieved. It is yet a further object of the present invention to 25 avoid the need of subjecting the material to be encapsulated to such harsh physical manipulations, and thereby reduce the possibility of inactivating or degrading that material.

It is also an object of the present invention to 30 provide a suitable method for encapsulating a wide variety of biologically active materials including, but not limited to, foods or nutritional substances as well as pharmaceutical agents, DNA, RNA, mRNA, nucleic acids, proteins, polypeptides, peptides and 35 enzymes. It is a further object of the present invention to provide a method which is simple,

- 6 -

feasible and inexpensive for the large-scale commercial production of liposomes and encapsulated materials.

5

SUMMARY OF THE INVENTION

The present invention includes a novel method for preparing liposomes and encapsulating a 10 biologically active material therein. The method involves: hydrating and mixing a quantity of lipid in an aqueous solution to form a liposome dispersion in the absence of an organic solvent or detergent; subjecting the liposome dispersion to one or more 15 cycles of freezing and thawing; and dehydrating the liposome dispersion to form a lipid powder. The lipid powder is suitable for both long-term storage and reconstitution to form liposomes and to encapsulate a biologically active material. The 20 lipid powder is hydrated in the presence of the biologically active material whereby the material is encapsulated in reconstituted liposomes. The method may optionally include the step of microfluidizing the reconstituted liposomes and/or the step of 25 separating encapsulated biologically active material from unencapsulated material.

The lipid can be a single or a combination of synthetic and natural lipid molecules. In addition, the method can include combining the liposome 30 dispersion with a bulking agent prior to dehydration and formation of the lipid powder. The weight to weight ratio of such a bulking agent to lipid is approximately 0.1:1 to 2:1.

The present invention provides a novel method 35 for encapsulating a wide variety of biologically active materials. There is no need to subject the

- 7 -

material to be encapsulated to harsh physical manipulations, and therefore, the possibility of inactivating or degrading that material is reduced. Moreover, the use of organic solvents or detergents 5 in forming the liposomes is advantageously avoided.

BRIEF DESCRIPTION OF THE DRAWINGS

10 FIGURE 1 depicts the improvement of lipid hydration upon the addition of a bulking agent.

FIGURE 2 depicts the SDS polyacrylamide gel (SDS-PAGE) migration pattern of liposome-encapsulated granulocyte colony stimulating factor.

15 FIGURE 3 depicts the release rate of encapsulated material in serum.

FIGURE 4 depicts the release rate of encapsulated calcein material in serum using different liposome compositions.

20 FIGURE 5 depicts the *in vivo* release rate of encapsulated material.

DETAILED DESCRIPTION OF THE INVENTION

25

The interest in liposomes as carriers of macromolecules is based on their ability to enclose and protect diverse materials and to deliver these materials, functionally intact and in significant 30 quantities, to large numbers of various cell types. The term liposome, as used herein, is intended to include any lipid bilayer structure consisting of closed concentric lamellae which enclose one or more aqueous-containing compartments.

35 The liposomes of the present invention are most frequently prepared from phospholipids, but other

- 8 -

molecules of similar molecular shape and dimensions having both a hydrophobic and a hydrophilic moiety can be used. For the purposes of the present invention, all such suitable liposome-forming 5 molecules will be referred to herein as lipids. One or more naturally occurring and/or synthetic lipid compounds may be used in the preparation of the liposomes.

Liposomes may be anionic, cationic or neutral 10 depending upon the choice of the hydrophilic group. For instance, when a compound with a phosphate or a sulfate group is used, the resulting liposomes will be anionic. When amino-containing lipids are used, the liposomes will have a positive charge, and will 15 be cationic liposomes.

Representative suitable phospholipids or lipid compounds for forming initial liposomes useful in the present invention include, but are not limited to, phospholipid-related materials such as 20 phosphatidylcholine (lecithin), lysolecithin, lysophosphatidylethanol-amine, phosphatidylserine, phosphatidylinositol, sphingomyelin, phosphatidylethanolamine (cephalin), cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, 25 phosphatidylcholine, and dipalmitoyl-phosphatidylglycerol. Additional nonphosphorous-containing lipids include, but are not limited to, stearylamine, dodecylamine, hexadecyl-amine, acetyl palmitate, glycerol ricinoleate, hexadecyl sterate, 30 isopropyl myristate, amphoteric acrylic polymers, fatty acid, fatty acid amides, cholesterol, cholesterol ester, diacylglycerol, diacylglycerolsuccinate, and the like.

In the present invention, a suitable lipid, or 35 lipid combination, is hydrated in an aqueous medium and mixed by any appropriate method including, but

- 9 -

not limited to, sonicating or vortexing the mixture, thereby forming the initial liposomes (e.g., dispersion of unilamellar vesicles). The liposome dispersion is formed in the absence of any organic
5 solvents or detergents. The liposome dispersion is then processed by one or more cycles of freezing and thawing (e.g., to form a suspension of multilamellar vesicles). For example, two to five repeated cycles of freezing and thawing can be advantageously used to
10 form the suspension. Following the freeze-thaw cycle(s), the liposome dispersion is dehydrated to form a lipid powder. The lipid powder may be further refined by any appropriate grinding or reducing process. The resultant lipid powder is particularly
15 stable and may be stored for extended periods making it feasible to produce bulk quantities which may be stored until needed. In a preferred embodiment, the liposomes are combined with a bulking agent prior to dehydration and formation of the lipid powder.

20 When a material is to be encapsulated, an appropriate amount of the lipid powder is combined with the material. The lipid powder is hydrated by any appropriate method and forms a mixture of unilamellar and oligolamellar vesicles ranging in size from 400-800 nm. For example, the combination
25 is hydrated by rotating the mixture on a rotovap apparatus or by stirring the mixture under vacuum. The hydrated lipid:material mixture is then optionally microfluidized in order to fully mix lipid and solute and provide encapsulation of solute. The
30 liposome-encapsulated biologically active material may then be separated from any remaining unencapsulated material. For example, the mixture may be centrifuged or dialyzed against distilled
35 water or buffered saline using a dialysis membrane.

- 10 -

As previously described, the present invention produces a mixture of unilamellar and oligolamellar vesicles. The determination that a mixture of vesicles is produced is based upon a comparison of 5 the actual trapped volume (ml solute/mmol lipid) with the theoretical trapped volume (calculated from the average diameter of the liposomes and the lipid concentration). Because the trapped volume was always slightly less than the theoretical volume, the 10 internal aqueous space of the liposomes must be smaller than that expected for a unilamellar liposome of the same diameter, and therefore, at least some of the liposomes must contain one or more lipid bilayers.

15 An important stage in the formation of liposomes is lipid hydration. The combined steps of the present invention promote full hydration of the lipid, and thus, full exposure of the lipid to the aqueous phase which contains the solute to be entrapped. The high extent of hydration leads to 20 highly efficient trapping of the aqueous phase. Freeze-thaw cycles are used in conjunction with a suitable mixing procedure to mix the lipid with and fully expose the lipid headgroups to the aqueous 25 phase. The FATMLV method, described above, produces both multilamellar and multivesicular liposomes with trapped volumes in the range of 1-6 μ l/ μ mole lipid. In contrast, the method of the present invention produces unilamellar and oligolamellar liposomes with 30 trapped volumes in the range of 20-30 μ l/ μ mole lipid.

The addition of a bulking agent prior to dehydrating the liposome dispersion accomplishes two important aspects of liposome production: ease of handling and rapid dispersal. The use of the bulking 35 agent leads to the production of a fluffy powder upon dehydration of the liposome dispersion. The

- 11 -

resultant lipid powder is much easier to handle than the waxy powder produced in the absence of a bulking agent. The bulking agent was also found to enhance the dispersal of the lipid in the dried state. This 5 characteristic decreases the tendency of the lipid to aggregate upon hydration and increases the contact of the aqueous phase with the lipid headgroups thereby enhancing both hydration and the capture of the solute.

10 Bulking agents suitable for use in the present invention include, but are not limited to, mannitol, sorbitol, lactose, glucose, sucrose, trehalose, glycine, arginine, gelatin, hydroxyethyl starch, albumin and xylitol. Other materials may be used if 15 their addition results in the production of a lipid powder which is easy to weigh and manipulate and which has an enhanced dispersion characteristic. The ratio of bulking agent to lipid is preferably about 0.1:1 to 2:1 wt/wt. Most preferably, the ratio is 20 approximately 1:1. The desired weight ratio is selected to enhance the stability of the liposome (e.g., the ratio is adjusted such that the liposome interior is not hyperosmotic relative to the external fluid, such as serum).

25 Microfluidization of the liposomes formed in accordance with the method of the present invention enhances the scale-up of liposome production. The microfluidizer (Microfluidics Corporation) uses an interaction chamber with a fixed geometry. Because 30 of this, the geometry of flow through the chamber is constant as long as pressure is constant. The manufacturer guarantees scale up ("linearly"), with the only limitation being the size of the pumping system.

35 The removal of any remaining unencapsulated material from the liposome-encapsulated material may

- 12 -

or may not be desired depending on the material and end use. If separation is appropriate, any suitable separation technique may be used. For example, the compositions may be separated by centrifugation, 5 dialysis, column chromatography and tangential flow filtration. The use of dialysis to remove unencapsulated material is a gentle procedure which does not lead to liposome leakage and can easily be scaled-up for commercial use.

10 It will be appreciated by those of skill in the art, that the individual mixing, dehydration, sizing and separation techniques employed in the performance of the present invention may include any suitable method. For example, while freeze-drying is a 15 convenient method of dehydration, other procedures such as vacuum drying, spray drying, and drying under a stream of nitrogen, would also be effective. Therefore, the specific techniques discussed herein or described in the individual steps of the following 20 Examples are not requirements of the present invention.

Major virtues of the present encapsulation technique, compared to other procedures, are its 25 mildness and simplicity. The present invention, in addition to providing high capture efficiency, is also quite gentle and leads to no detectable aggregation or degradation of the materials encapsulated. The method is therefore advantageously used to encapsulate biologically active materials 30 which are sensitive to one or more of the manipulations required by conventional liposome formation and encapsulation procedures. The present invention has been used successfully for encapsulating granulocyte-colony stimulating factor 35 (G-CSF), consensus interferon (IFN), porcine growth hormone (pGH) and antisense oligonucleotides.

- 13 -

Encapsulation of up to 80-90% of input G-CSF and IFN has been achieved. Greater than 40% of input oligonucleotide has been encapsulated. The present invention would be suitable for encapsulating 5 cytokines of the general structure described in Hill, et al (PNAS 90: 5167-5171; 1993). These cytokines include, but are not limited to, GM-CSF, M-CSF, hGH, pGH and IL-2 which are relatively unstable proteins (especially G-CSF and pGH). The method of the 10 present invention, however, is gentle enough to accommodate these materials without degradation.

Unique to the method of the present invention are the combined techniques of forming a liposome dispersion, subjecting the liposome dispersion to one 15 or repeated freeze-thaw cycles followed by dehydration, and performing these sequential steps without combining the material to be encapsulated with the liposome dispersion. Thus, in the present invention, the liposomes are formed, their volumes 20 are maximized and a storable lipid powder is produced for later reconstitution and the encapsulation of the desired material. Prior to the present invention, it was not previously known that the advantageous results of the freeze-thaw cycles would survive the 25 dehydration of the liposome dispersion as well as the subsequent hydration of the lipid powder with the biologically active material. Nor was it appreciated that the combined steps, completed in a preferred order, could be performed in the absence of the 30 material to be encapsulated. In addition, there is no need for an organic solvent or other chemical manipulations, the presence of which may degrade or destroy the material to be encapsulated. Furthermore, a labile material, such a biologically 35 active material, is not subjected to the extreme

- 14 -

physical manipulations involved in producing the lipid powder.

The present invention has several additional advantages over previously described encapsulation methods and several unexpected outcomes. One problem encountered in previous liposome preparation methods is batch-to-batch variations. In the present invention, however, dried powders of the appropriate lipids can be prepared beforehand in large batches and stored (for example, under nitrogen at -20°C) until the time of use. Unexpectedly, the method also leads to the formation of large liposomes exhibiting high aqueous capture, and does so without exposing the encapsulated material to stressful manipulations (e.g., sonication) or organic solvents. The liposomes produced are larger than those produced by conventional methods (such as described in U.S. Patent No. 4,735,788), and therefore, the liposomes have a larger aqueous compartment and a higher trapping efficiency. It was also unexpected that dehydration of the resultant liposome dispersion to form a lipid powder and the later reconstitution of the liposomes from that lipid powder would produce liposomes having an enhanced encapsulation efficiency as found in the present invention and as confirmed in the following Examples.

Liposomes prepared using the present invention also have a high degree of stability (e.g., low leakage of entrapped contents) and are useful as sustained release depots for *in vivo* delivery of biologically active materials. Biologically active materials encapsulated in accordance with the methods of the present invention exhibit a slow release from the liposome over several days following subcutaneous injection.

- 15 -

EXAMPLES

The following abbreviations are used in the

5 Examples:

conIFN: consensus human interferon
GM-CSF: recombinant human granulocyte-macrophage
colony stimulating factor
pGH: porcine growth hormone
rhG-CSF: recombinant human granulocyte colony
stimulating factor
CHOL: cholesterol
DM: dimyristoyl (di-14:0)
DP: dipalmitoyl (di-16:0)
DS: distearoyl (di- 18:0)
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PG: phosphatidylglycerol

Example 1

Comparison of four methods for encapsulation

10

The following analysis examined the results of conventional liposome preparation and encapsulation methods (Methods 1 and 2) as compared to the methods of the present invention (Methods 3 and 4). The 15 biologically active material was recombinant human granulocyte colony stimulating factor (rhG-CSF).

Method 1: Chloroform stocks of DMPG, DSPC and CHOL were combined to achieve a mole ratio of 1:4:5. The 20 lipid mixture was dried under N₂ (g) and desiccated

- 16 -

for one hour under vacuum to form a lipid film. The total amount of lipid used was 300 μ moles. The lipid was then hydrated for one hour in rhG-CSF (10 ml of 4 mg/ml in dilute HCl, pH 4.5) at a temperature above 5 the chain melting temperature (T_m) of DSPC (60°C). The samples were then microfluidized at 10,000 psi for 10 cycles using a Microfluidics model 110S instrument (Microfluidics Corporation; Newton, MA 02164). Unencapsulated G-CSF was removed from the 10 resultant liposomes by pelleting the liposomes at 100,000 xg using a Beckman air driven ultracentrifuge. The amount of encapsulated G-CSF was determined using the BCA protein assay (Pierce Chemical Company; Rockford, Illinois).

15 Method 2: Chloroform stocks of lipid were combined, dried and desiccated as described in Method 1. The lipid film was hydrated in distilled water (10 ml, 60°C) and sonicated (lipid concentration of 30 mM) 20 for ten minutes in a bath-type sonicator (Laboratory Supplies, Hicksville, NY). The hydrated lipid was freeze-dried and then ground using a mortar and pestle. The ground lipid was hydrated in G-CSF and microfluidized, as described in Method 1. Separation 25 of unencapsulated G-CSF from encapsulated G-CSF was achieved by dialysis of the samples against water (16 l), using a dialysis membrane with a 100,000 MW cutoff. Encapsulated G-CSF was quantified using the BCA protein assay as in Method 1.

30 Method 3: Lipid films, prepared as described in Method 1, were hydrated in water and sonicated. The sonicated dispersions were then subjected to 3-5 cycles of freezing (dry ice/acetone) and thawing 35 (37°C). The dispersions were freeze dried and ground as in Method 2 to form a lipid powder. The powder

- 17 -

was subsequently hydrated in G-CSF, and the liposome-encapsulated material was microfluidized as described in Method 1. Unencapsulated G-CSF was separated from encapsulated G-CSF as described in Method 2.

5

Method 4: The lipid samples were prepared, sonicated and subjected to freeze/thaw cycles as described in Method 3. Prior to dehydrating the sample, D-mannitol was added (1:1 weight ratio to lipid) as a 10 bulking agent. The samples were freeze-dried and rehydrated, upon use, as described in Method 3. Hydration and microfluidization was carried out as described in Method 1. The samples were then dialyzed as described in Method 2.

15 A comparison of the results of the various production methods is presented in Table 1. The data illustrate the physical characteristics of the storable powder as well as the capture efficiency and capture volume of the liposomes which were produced.

20

Table 1
Encapsulation of rhG-CSF

<u>Method</u>	<u>Encapsulation</u> (% of G-CSF input)	<u>Trapped Volume</u> (μ l/ μ mole lipid)	<u>Powder Obtained</u> (after freeze- drying)
1	2%	0.6	NA
2	30%	10	waxy
3	80%	26.6	waxy
4	90%	30	fluffy

25 Method 4 was applied to a variety of other biologically active materials to further assess the suitability of the liposome preparation and

- 18 -

encapsulation method. The results presented in Table 2 illustrate that the present invention produces a liposome preparation capable of highly efficient encapsulation.

5

Table 2
Encapsulation of various protein therapeutics and
oligonucleotides using Method 4.

<u>Lipid Composition</u>	<u>Solute</u>	<u>% of Input Encapsulated</u>	<u>Trapped Volume</u> (μ l/ μ mole total lipid)
DSPC:DSPE (9:1)	con-IFN	88	29.3
DMPC:CHOL (3:1)	con-IFN	71	21.3
DMPG:DSPC:CHOL (1:4:5)	G-CSF	90	30
DPPC	G-CSF	85	28.3
DPPC	oligonucleotide (anti-sense c-myb)	45	22.5
DPPC	GM-CSF	97	29.1
DPPC	pGH	87	30.3

10

Example 2

Addition of a bulking agent to enhance lipid hydration and capture

15

Method 3 and Method 4 (above) were compared with respect to lipid hydration, a key phase in liposome formation and material capture. Samples of DMPG:DSPC:CHOL (1:4:5 molar) were prepared as 20 described above, and a bulking agent (e.g., D-

- 19 -

mannitol) was added to some samples at a weight approximately equal to the total lipid weight. The samples were dehydrated and then rehydrated in G-CSF as described above. Hydration was carried out at 5 37°C at a lipid concentration of 30 mM for the times indicated in the Figure 1. Aliquots of the liposome suspensions were assayed for lipid content using a phosphate assay (Bartlett, et al., *J Biol Chem* 234: 466-468; 1959).

10 Figure 1 depicts the percent recovery of material with or without the addition of a bulking agent. The addition of the bulking agent improved lipid hydration and the formation of liposomes. By improving lipid hydration, more liposomes were formed 15 in a given volume and more of the aqueous phase was captured.

Example 3

Stability of encapsulated material

20 The following procedure illustrated that the method of the present invention advantageously failed to elicit the aggregation or degradation of 25 encapsulated materials, even materials quite sensitive to physical and chemical manipulations.

G-CSF, either alone or encapsulated in DMPG:DSPC:CHOL (1:4:5) liposomes was run on 10-20% SDS gel under non-reducing (lanes 4 & 5) and reducing 30 (lanes 1 & 2) conditions. Fifty nanograms of G-CSF was run per lane, and the protein was detected by silver stain. The results of SDS-PAGE of the encapsulated G-CSF are illustrated in Figure 2. (Lanes 1 and 5: G-CSF. Lanes 2 and 4: liposome-35 encapsulated G-CSF. Lane 3: MW markers.) The results demonstrate that liposome encapsulation does

- 20 -

not lead to aggregation of the encapsulated material. In addition, the method is gentle and leads to no detectable destruction or loss of activity of the biologically active material.

5

Example 4

Reverse phase HPLC of encapsulated G-CSF

10 Biologically active material encapsulated in accordance with Method 4, above, was also subjected to reverse phase high pressure liquid chromatography (HPLC) to determine whether liposome-encapsulation led to structural changes in the encapsulated material. For example, G-CSF alone or encapsulated in DMPG:DSPC:CHOL (1:4:5) liposomes was examined by C4 reverse phase HPLC under the following conditions:

Column: C-4 silica
Buffer A: 0.1% trifluoroacetic acid in water
20 Buffer B: 0.1% trifluoroacetic acid in 90% acetonitrile
Gradient: 0-90% B in 60 minutes
Flow rate: 0.8 milliliter/minute
Detection: 220 nanometers

25 Thirty micrograms of G-CSF was loaded per run. Liposomes were lysed in 40% methanol for 30 minutes at 37°C prior to chromatography.

30 The results are depicted in Table 3 which illustrates that the G-CSF released from liposomes is structurally identical to G-CSF which was never encapsulated. While protein fragments and aggregates are easily detected on HPLC as discrete peaks, the control G-CSF and the G-CSF released from the lysed liposomes produced nearly identical peaks. These 35 results illustrate that there was no material

- 21 -

destruction or aggregation of G-CSF upon encapsulation.

Table 3

5 Reverse Phase Chromatography of Liposome-encapsulated G-CSF

<u>Sample</u>	<u>Amount injected</u> (μ g G-CSF)	<u>Retention Time</u> (minutes)	<u>Recovery</u> (%)
G-CSF	30	39.6	100
DMPG:DSPC:CHOL (1:4:5)	30	39.7	100

Example 5

Sustained release of encapsulated material.

Liposomes prepared in accordance with the method of the present invention exhibit a slow release of encapsulated contents. Sustained release of encapsulated material was found in serum and *in vivo* after subcutaneous injection.

Calcein release: Liposomes (DPPC; DMPG:DSPC:CHOL; 20 DSPC:CHOL) were prepared substantially in accordance with Method 4, above, to contain calcein, a self-quenching fluorescent dye (MW 622). The liposomes were diluted into 80% human serum and incubated at 37°C. The final lipid concentration during the 25 incubation was 6 mM. At the indicated times, aliquots of the samples were removed and diluted to 12 μ M (1:500) or less. Fluorescence was measured before (F) and after (F_T) addition of Triton X-100 to a final concentration of 1%. The percent release was

- 22 -

calculated as: %Release = $100(F - F_0)/(F_T - F_0)$ where F_0 is the fluorescence at 0 time (or no serum).

Figure 3 depicts the release of calcein in 80% serum at 37°C. The results demonstrate that 5 liposomes with slow release characteristics are prepared in accordance with the present invention. Liposomes having such characteristics are useful for sustained release of biologically active materials *in vivo*.

10 Various liposome compositions were also evaluated for the leakage or release of encapsulated material. Figure 4 depicts the varying rates of release achieved (release of calcein *in vitro*) using different lipid compositions in the production of the 15 liposomes of the present invention.

In vivo release: Liposomes composed of either DPPC or DMPG:DSPC:CHOL (1:4:5 molar) were prepared substantially in accordance with Method 4, above, to 20 contain either ^{125}I -tyramanyl inulin (at a final specific activity of 0.4×10^6 dpm/ml) or ^{125}I -rhG-CSF (at a final specific activity of 1×10^6 dpm/ml). (See: ^{125}I -labeled Inulin: A convenient marker for deposition of liposome contents *in vivo*. Sommerman, 25 E.F., Pritchard, P.H. and Cullis, P.R. *Biochem Biophys Res Commun* Vol. 122(1), 319-324; 1984.) The final lipid composition was 30 mM. Sprague-Dawley (S. D.) rats were injected subcutaneously with the lipid compositions (0.5 ml) indicated in Figure 5. 30 The injection site was marked. At various times after injection, the rats were sacrificed and the injection site was excised and counted for remaining ^{125}I . Percent release was defined as %Release = $100(1 - \text{DPM}_t/\text{DPM}_0)$, where DPM_t is the dpm remaining at 35 the site at time t , and DPM_0 is the initial dpm injected ($t=0$).

- 23 -

Figure 5 depicts the *in vivo* release of rhG-CSF and inulin following subcutaneous injection of these liposome-encapsulated materials into rats. DPPC liposomes were found to release fairly rapidly *in vivo* in comparison to DMPG:DSPC:CHOL which exhibited slower release kinetics. No significant difference in the leakage of inulin or G-CSF was noted for either liposome composition.

Those skilled in the art will appreciate that a variety of pharmaceutically acceptable carriers can be used to facilitate the administration of the liposomes of the present invention. The selection of a suitable carrier may also be determined in accordance with the desired route of administration.

15

CLAIMS

What is claimed is:

- 5 1. A method for preparing liposomes and encapsulating a biologically active material therein, comprising the steps of:
 - a) hydrating a quantity of lipid in an aqueous solution and mixing to form a liposome dispersion
 - 10 b) subjecting said liposome dispersion to one or more cycles of freezing and thawing;
 - c) dehydrating said liposome dispersion to form a lipid powder; and
 - 15 d) hydrating said lipid powder in the presence of the biologically active material whereby said biologically active material is encapsulated in reconstituted liposomes.
- 20 2. The method according to Claim 1, further comprising the step of microfluidizing said reconstituted liposomes.
- 25 3. The method according to Claim 1, further comprising the step of separating encapsulated biologically active material from unencapsulated biologically active material.
- 30 4. The method according to Claim 1, further comprising combining said liposome dispersion with a bulking agent prior to dehydration and formation of said lipid powder.
- 35 5. A method of preparing liposomes and encapsulating a biologically active material therein, comprising the steps of:

- 25 -

- a) preparing a dispersion of unilamellar vesicles (SUV) by bath sonication of a lipid in the presence of an aqueous solvent in the absence of an organic solvent or detergent;
- 5 b) subjecting said SUV to one or more freeze-thaw cycles to form a suspension of multilamellar vesicles (MLV);
- c) combining said MLV suspension with a bulking agent, wherein the lipid:bulking agent weight to
- 10 weight ratio ranges from 0.1:1 to 2:1;
- d) freeze-drying said mixture of MLV and bulking agent to form a lipid powder;
- e) hydrating said lipid powder in a solute to be entrapped and microfluidizing said hydrated lipid,
- 15 whereby said solute is encapsulated in reconstituted liposomes; and
- g) separating unencapsulated solute from said liposomes.

20 6. The method according to Claim 5, wherein said hydrated lipid powder is microfluidized for 10 cycles at 10,000 psi.

25 7. The method according to Claim 5, wherein unencapsulated solute is separated from said liposomes by dialysis against an appropriate aqueous solvent using a dialysis membrane with at least a 100,000 MW cutoff.

30 8. A liposome-encapsulated biologically active material produced by:

- a) hydrating a quantity of lipid in an aqueous solution and mixing to form a liposome dispersion in the absence of an organic solvent or detergent;
- 35 b) subjecting said liposome dispersion to one or more cycles of freezing and thawing;

- 26 -

- c) dehydrating said liposome dispersion to form a lipid powder; and
- d) hydrating said lipid powder in the presence of a biologically active material whereby said

5 biologically active material is encapsulated in reconstituted liposomes.

9. The liposome-encapsulated material according to Claim 8, further comprising a bulking

10 agent added prior to dehydration and formation of said lipid powder.

10. The liposome-encapsulated material according to Claim 8, wherein said lipid is a

15 combination of synthetic and natural lipid molecules.

11. A method for preparing a lipid powder suitable for the formation of liposomes, comprising the steps of:

- 20 a) hydrating a quantity of lipid in an aqueous solution and mixing to form a liposome dispersion in the absence of an organic solvent or detergent;
- b) subjecting said liposome dispersion to one or more cycles of freezing and thawing; and
- 25 c) dehydrating said liposome dispersion to form a lipid powder;

 wherein said lipid powder is suitable for storage and suitable for reconstitution to form liposomes and encapsulate a material by hydrating said lipid powder

30 in the presence of said material.

12. The method according to Claim 11, wherein said lipid is a combination of synthetic and natural lipid molecules.

FIG. I

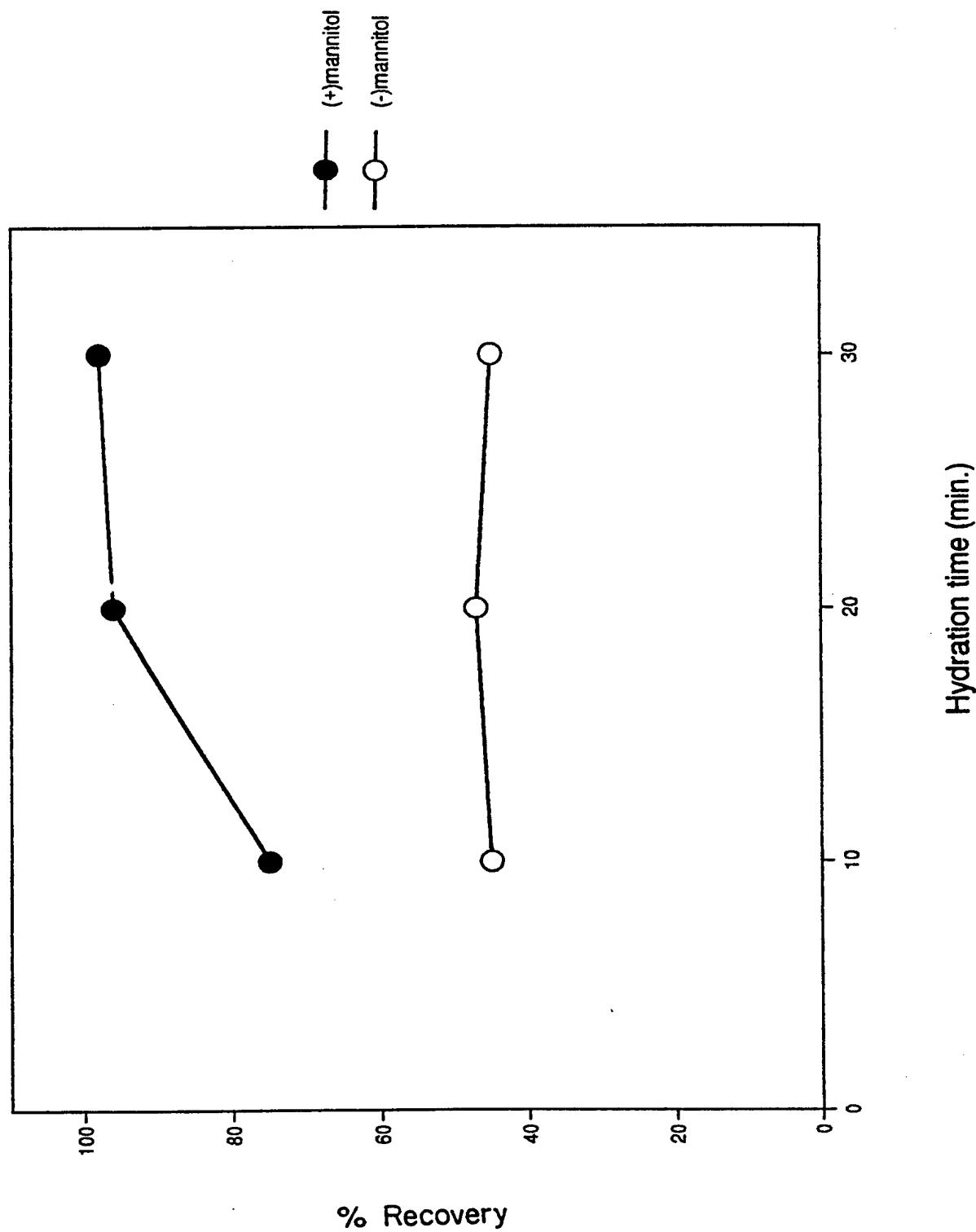


FIG. 2

1 2 3 4 5

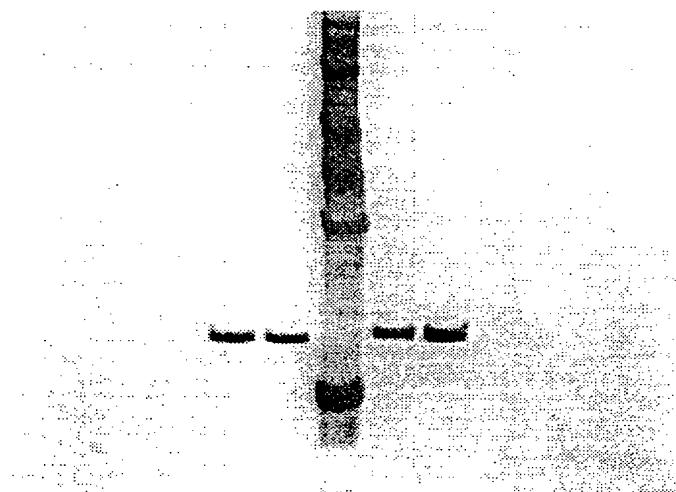


FIG. 3

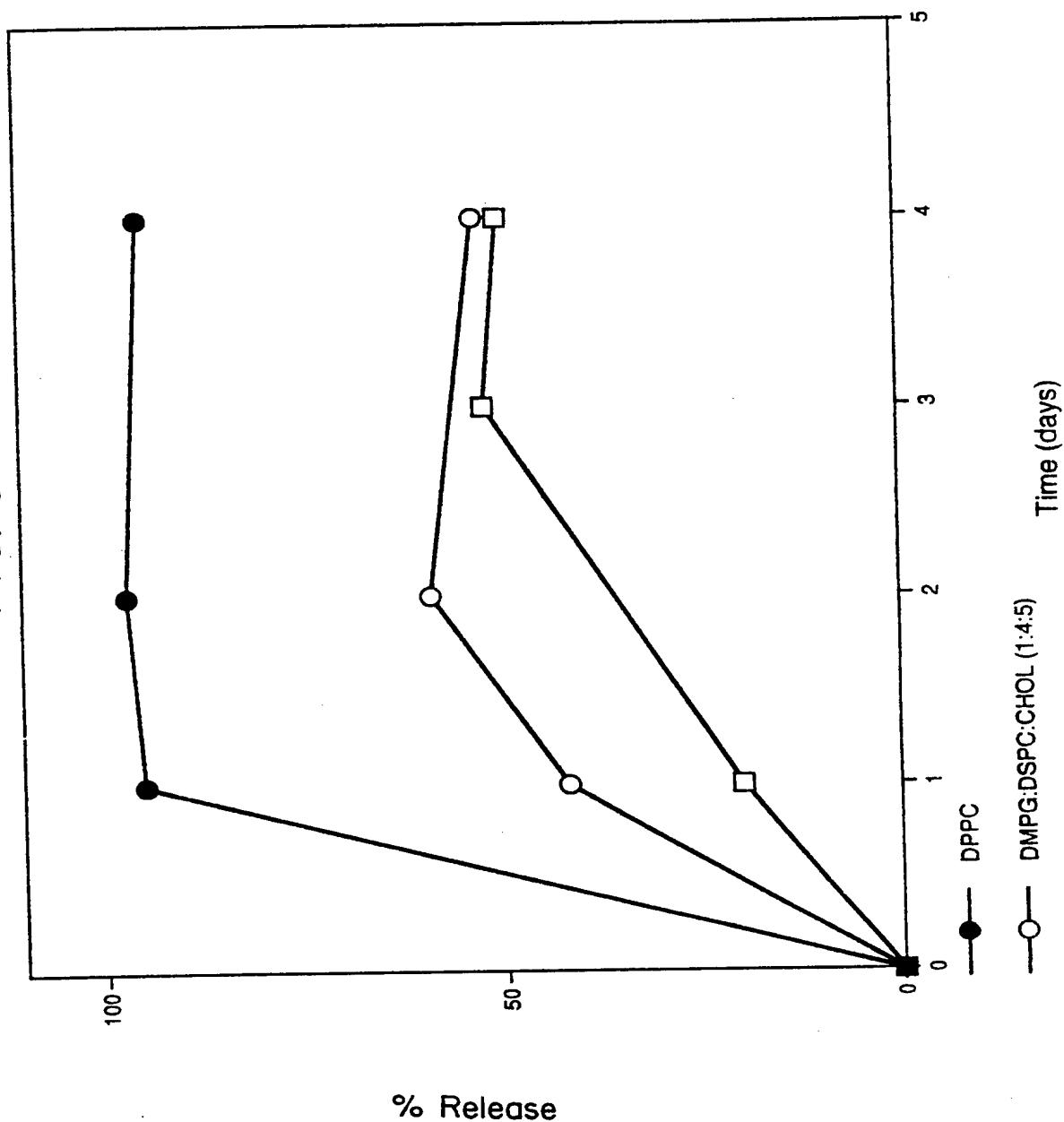


FIG. 4

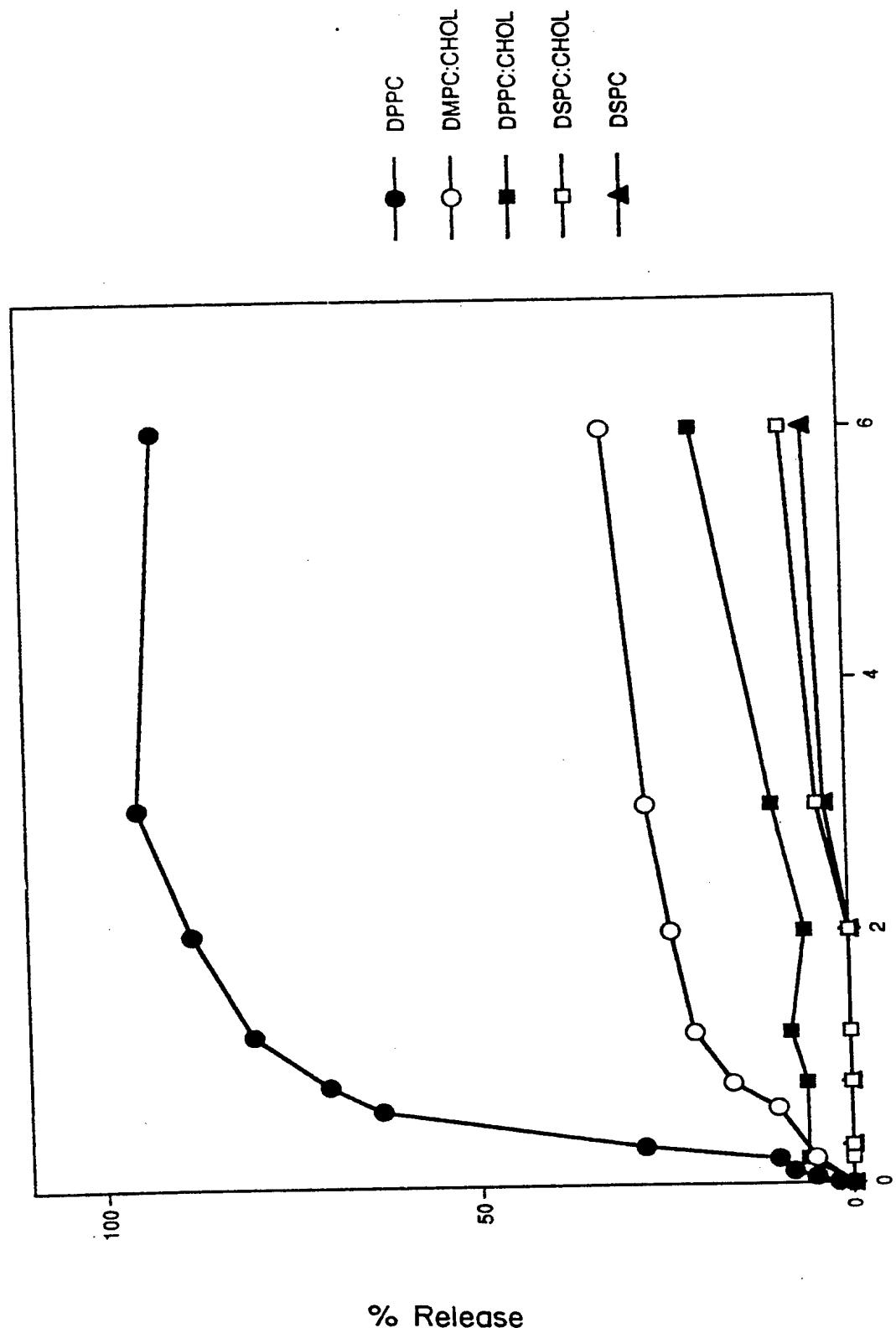
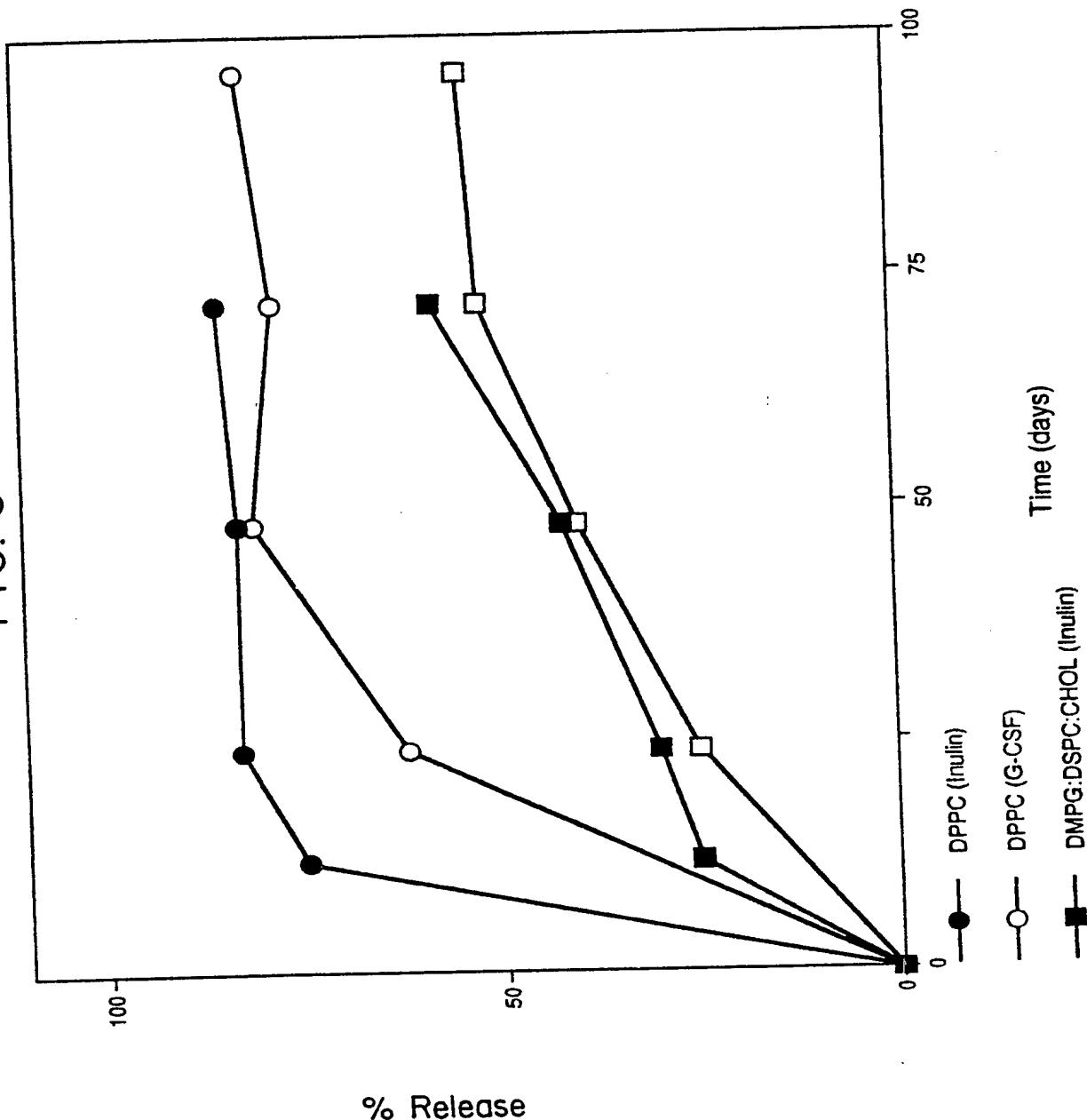


FIG. 5



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/12350

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CHEM. PHARM. BULL., vol.37, no.7, July 1989, TOKYO (JP) pages 1895 - 1898</p> <p>H. JIZOMOTO ET AL. 'encapsulation of drugs by lyophilized, empty dipalmitoylphosphatidylcholine liposomes' see the whole document see page 1895, column 2, paragraph 2 see page 1897, column 2; table v ---</p>	1-12
A	<p>NUCLEIC ACIDS RESEARCH, vol.19, no.20, 25 October 1991, EYNSHAM, OXFORD (GB) pages 5551 - 5559</p> <p>S. AKHTAR ET AL. 'interactions of antisense dna oligonucleotide analogs with phospholipid membranes (liposomes)' see page 5553, column 2, paragraph 1 ---</p>	1-12 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

1 Date of the actual completion of the international search

15 March 1995

Date of mailing of the international search report

28.03.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Benz, K

INTERNATIONAL SEARCH REPORT

Internal Application No

PCT/US 94/12350

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	D. GLICK 'methods of biochemical analysis, vol. 3' 1988 , JOHN WILEY & SONS , NEW YORK (US) see page 428, paragraph B. - page 431, paragraph C. ---	1-12
A	JOURNAL OF MICROENCAPSULATION, vol.8, no.4, October 1991, LONDON (GB) pages 547 - 562, XP232589 J.-C. VUILLEMARD 'recent advances in the large-scale production of lipid vesicles for use in food products: microfluidization' see page 557 -----	2,6